Radiosensitization of Normal Human Cells by LY294002: Cell Killing and the Rejoining of DNA and Interphase Chromosome Breaks

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LY294002/DNA double strand break/Repair/Chromosome/Radiation.

The radiosensitizing effect of a phosphoinositide-3 kinase (PI3K) inhibitor, wortmannin, has been studied rather extensively, but there have been few studies on the radiosensitizing effect of another PI3K inhibitor, LY294002. In this report, we present the radiosensitizing effect of LY294002 using normal human cells. Clonogenic cell survival indicated that LY294002 enhanced the killing effect of γ-rays in a dose-dependent manner, although this by itself did not affect the cell killing. To obtain a 10% cell survival, about one half of the radiation dose was needed when cells were treated with 50 μM LY294002 as compared to cells without the drug. A mild inhibition of repair of DNA double strand breaks (DSBs) was observed in irradiated normal human cells pre-treated with LY294002 (50 μM). At the interphase chromosome level, we also observed an increase in the number of residual breaks when irradiated cells were pre-treated with this drug (about 2-fold at 5 Gy). These results suggest that the inhibition of DSB repair mediated the radiosensitizing effect of LY294002 at the dose level that we used.

INTRODUCTION

Wortmannin and LY294002 are two widely used inhibitors of phosphatidylinositol 3-kinase (PI3K), an important enzyme for intracellular signaling.1,2) Wortmannin, a fungal metabolite, was isolated as an anti-inflammatory drug3) and LY294002, which is not structurally related to wortmannin, was developed as a selective inhibitor of PI3K.4) Due to the similarity between the kinase domain of PI3K and the genes responsible for radiation hypersensitivity such as ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs, now known as prkdc),5,6) the radiosensitizing effect of wortmannin was studied in various mammalian systems.6-13) An additional study also revealed the radiosensitization effect of LY294002 using human tumor cells.14) Both Wortmannin and LY294002 inhibited the DNA-PK function in vitro and in vivo.14-16) Several studies indicated that the wortmannin sensitization effect most likely results from the repair inhibition of radiation induced DNA double strand breaks (DSBs), specifically non-homologous end joining (NHEJ).13,14,15) NHEJ is known to be one of the major DNA DSB repair pathways in mammalian cells.16,17) Using in vitro cell-free systems, the inhibitory effect of wortmannin and LY294002 on the rejoining of broken DNA ends has been shown.16,21)

In spite of the significant drug actions reported for both wortmannin and LY294002, much less is known of the molecular target for the radiosensitization effect. Izzard et al.18) have shown that wortmannin binds to the kinase domain of DNA-PKcs in vitro and in vivo. This is consistent with the suggested wortmannin binding site, amino acid residue 3751 in DNA-PKcs, which has been obtained from an analogy of the wortmannin target in PI3K.21) The target molecule for wortmannin radiosensitization being DNA-PKcs is consistent with several other studies.9,13)

We previously demonstrated that wortmannin radiosensitizes normal human fibroblasts by inhibiting the repair of DNA DSB induced by gamma-radiation, and the target of wortmannin action might be at the C-terminus region of DNA-PKcs protein.9) Since no DNA DSB repair study in vivo (cells) is available with LY294002 and a recent report suggested that LY294002 radiosensitized only cells with mutant ras oncogenes,21) we wanted to clarify the key issues associated with this drug using normal human fibroblasts. Our data revealed a clear radiosensitizing effect of LY294002 (at 50

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μM or higher) in normal human cells as the result of a mild repair inhibition of gamma-ray induced DNA DSB leading to an excess number of remaining interphase chromosome breaks.

**MATERIALS AND METHODS**

**Cells and irradiation**

The normal human fibroblast cell line GM08399 was obtained from Coriell Cell Repositories (Camden, NJ) and maintained in alpha-MEM medium containing 15% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO) and antibiotics. Exponentially growing cells were used for most experiments. Irradiations were carried out using a 137Cs γ irradiator at a dose rate of 3.90 Gy/min. For cell survival experiments, irradiated cells were trypsinized, counted and replated so that each dish would yield 50–200 colonies after 10–14 days of incubation. LY294002 or DMSO (for control) was added (from 50 mM stock solution dissolved in DMSO) to dishes 45 min before irradiation. Sixteen hours after irradiation and plating dishes in LY294002 medium, the medium was replaced with LY294002 free medium. Colonies containing more than 50 cells were counted after having been stained with 10% Giemsa. The radiation cell survival curve was normalized to that for unirradiated controls with DMSO or appropriate concentrations of LY294002. The maximum DMSO concentration in the medium was 0.2%. The plating efficiency for control population was 25–35% with this cell line.

**DNA double strand break (DSB) repair assay**

Constant field gel electrophoresis (CFGE) described by Dahm-Daphi et al. was used with a slight modification. To quantify the level of DSBs, cells were incubated with 370 Bq/ml 3H-TdR (Thymidine) and 5 μM cold TdR for at least 2 days before each experiment. Cells were irradiated (20 Gy) in dishes and then incubated in a CO2 (5%) incubator at 37°C for repair. At each point, cells were trypsinized, washed in cold medium, and the resulting cell pellet was immediately embedded in 0.6% agarose (Incert agarose, FMC). These agarose samples were cut into plugs and immersed in an ice-cold lysis solution containing 0.5M EDTA, 0.01 M Tris, 2% Sarkosyl and 0.2 mg/ml proteinase K (pH 8.0) for 1 h and then incubated overnight at 50°C. For the initial time point (0 h), cells were embedded in agarose and irradiated in ice cold medium followed by an immediate lysis as described. After lysis, samples were washed for 1 h in 0.1 M EDTA, 0.01 M Tris at pH 8.0, and treated with 0.1 mg/ml ribonuclease A for 1 h at 37°C.

Electrophoresis was performed in 0.5× TBE buffer (45 mM Tris, 45 mM Boric Acid, 1.5 mM EDTA, pH 8.2) with 0.6% agarose gel (Gibco BRL) at room temperature in a conventional gel box for 40 h at a constant field strength of 0.6 V/cm. After electrophoresis, gels were stained with ethidium bromide, photographed under UV light, and cut to separate the plug from the lane of each sample. The 5°C activity of each sample was measured in a scintillation counter and the fraction of activity released (FAR) was calculated as the disintegration per min (DPM) of a lane divided by the total DPM (lane + plug) per sample. The FAR value for non-irradiated control cells was 1–3%, which was subtracted from the values for the irradiated samples.

**Premature chromosome condensation assay**

Cell fusion mediated by sendai virus was used in obtaining interphase chromosome preparations.25-27 Confluent cultures of GM08399 cells were exposed to LY294002 for 45 min and irradiated with 5 Gy gamma-rays. After 4 h of repair incubation at 37°C, cells were trypsinized, counted and one million GM08399 cells were mixed with one million HeLa mitotic cells. The mitotic cells were prepared beforehand by double thymidine block (mitotic index >97%) and kept frozen at –80°C until the time of fusion. The mixed sample was washed once in cold serum free medium and pelleted. 100 hemagglutinating unit (HAU) of UV inactivated sendai virus was added to the pellet along with 0.7 ml cold serum-free medium with colcemid (0.1 μg/ml final concentration) and the mixed sample was incubated for 15 min in ice for virus attachment. Subsequently, the sample was incubated at 37°C for 70 min under an adjusted pH. After incubation, the sample was treated with a hypotonic (0.075 M KCl) solution for 15 min at 37°C, fixed in methanol:acetic acid (3:1) twice and stained with 5% Giemsa solution. The stained PCC samples were analyzed using bright-field microscopy. At least 50 PCC spreads were scored for each condition (control, LY294002 alone, 5 Gy γ-ray alone and LY294002 plus 5 Gy γ-ray).

**RESULTS**

The gamma-ray sensitivity of log-phase normal human fibroblasts (GM 08399) pre-treated with various concentrations of LY294002 was examined by colony formation. The results are presented in Fig. 1. Although the radiosensitizing effect of LY294002 was dependent on the drug concentration, the sensitizing effect was saturated for concentrations above 50 μM. 50 μM LY294002 was used by another laboratory in a cancer cell line and a similar level of radiosensitization was observed.16 The similar radiosensitization for cancer and normal cells would suggest that tumor cells are not inherently more prone to sensitization by LY294002 than normal cells. Another PI3K inhibitor, wortmannin, gave a significantly higher radiosensitization effect with a lower drug concentration (20 μM).28 The 50 μM LY294002 alone displayed minimal cytotoxicity (<10% cell killing with the drug alone), and this toxicity is a little lower than 20 μM wortmannin (12–30%) in the same cell line.9 In order to obtain 10% cell survival, 1.9 to 2.0 times less radiation dose was required in cells pretreated with 50 μM LY294002 when compared with cells
Fig. 1. Gamma-ray cell survival curves in exponentially growing normal human fibroblasts (GM08399) pretreated with various concentrations (5, 50 and 100 μM) of LY294002. Cells were treated with LY294002 for 45 min before irradiation, trypsinized, and re-plated with appropriate cell numbers for colony formation; the medium was changed 16 h later. These curves were obtained from two to three independent experiments and the mean and the standard errors are given for each point.

Fig. 2. Kinetics of DNA DSB rejoining in gamma-irradiated (20 Gy) exponentially growing GM08399 cells pretreated with 50 μM LY294002 as compared to cells with radiation alone (control). For a comparison, the rejoining kinetics of the irradiated cells pretreated with 20 μM wortmannin is indicated by a dotted line. The background FAR value was subtracted from each point. These results were obtained from two to three independent experiments; the mean and the standard errors are given.

without the drug.

The effect of DNA DSB rejoining was studied by constant field gel electrophoresis (CFGE) and the results (normalized at 0 h repair point) are presented in Fig. 2. CFGE was used because the sensitivity is comparable to that of the pulsed-field gel electrophoresis assay, and no major equipment was necessary with this method. The rejoining of radiation-induced DNA DSB was clearly inhibited by 50 μM LY294002 as compared to control samples without any drug. It appears that the repair inhibition was more pronounced in the initial part of rejoining (0–1 h). Our DSB repair inhibition data by LY294002 in cells are consistent with the data obtained from cell-free in vitro systems. As compared with wortmannin (20 μM), with which the remaining damage at 4 h repair point was higher than 50% of the initial DSB in the same cell line, the repair inhibition by LY294002 is considerably milder with 20–30% residual breaks at the 4 h point.

In order to extend the DNA DSB inhibition data in Fig. 2, we initiated studies at the chromosome level using the premature chromosome condensation (PCC) assay. For this purpose, confluent (G0/G1) cultures of GM08399 cells were treated under various conditions. These cells were then fused with mitotic HeLa cells by means of UV inactivated sendai viruses. Since our exponential population consisted of 60–80% G1 phase cells, the PCC data with the confluent culture would provide a good representation for exponential cells. The resultant G1 type PCC was scored for the four data points including (i) no drug/no radiation control, (ii) 50 μM LY alone, (iii) 5 Gy radiation alone with 4 h repair, and (iv) 50 μM LY plus 5 Gy gamma-rays with 4 h repair. The summarized results with PCC are given in Fig. 3 with a histogram of the repaired samples. As expected, the control sample had 46.1 chromosomes per cell and an addition of LY294002 did not produce a significant increase in the excess PCC fragments when compared with the control. In contrast, when combined with 5 Gy gamma-rays, LY294002 rendered an
excess number of chromosome fragments almost two fold (3.7 excess breaks with 5 Gy alone versus 6.7 excess breaks with LY294002 plus 5 Gy). Thus, the repair inhibitory effect of LY294002 is obvious at the chromosome level. A statistical analysis on these values shows that the difference (3.7 vs. 6.7) is highly significant ($p < 0.001$). These values correlate well with the sensitizer enhancement factor of about 2, calculated from the cell survival data in Fig. 1. Since DNA DSBs are thought to underlie PCC breaks,$^{[26][30][31]}$ our PCC results are consistent with the DNA DSB repair inhibitory effect of LY294002 at the biologically significant dose range.

**DISCUSSION**

As in the case of wortmannin, we have shown that LY294002 radiosensitizes normal human cells and inhibits the repair of DNA DSBs at the dose we used (50 $\mu$M). The repair inhibition was also indicated by the reduced rejoining of interphase (G1) chromosome breaks, judging from excess residual fragments by the PCC method. Since G1-type chromosomes were significantly affected, the type of DSB repair influenced by LY294002 would be the NHEJ type. The degree of repair inhibition by LY294002 was less than that caused by wortmannin. To our knowledge, this is the first report of LY294002 being an inhibitor of DNA DSB and chromosome break rejoining in cells (in vivo). As mentioned, the repair inhibitory effect of this drug in vitro (cell free system) has been documented,$^{[16][21]}$ but no chromosome study with LY294002 combined with radiation has been published.

Although LY294002 is a good radiosensitizer with no significant cytotoxicity and chromosome damage by itself, it does not seem to show a differential effect between tumor and normal cells. Normal cells are sensitized to the same degree by this drug as are tumor cells.$^{[14]}$ Nevertheless, LY294002 or a similar drug might be useful in radiation therapy if there is a selective uptake of the drug in tumor tissues, or if radiation is largely confined to the tumor (e.g., stereotactic radiosurgery$^{[22][32]}$).

We have tried to determine the target of LY294002 radiosensitization using Western blot as we did in the wortmannin study; however, no unequivocal result was obtained. For some experiments, different band patterns were observed with an antibody detecting the C-terminus end of DNA-PKcs when cells were treated with LY294002, although we could not confirm this phenomenon. Thus, it seems that the action of LY294002 might be different from wortmannin which could affect the C-terminus end of DNA-PKcs molecule.

As mentioned in the introduction, a recent study showed that only cells bearing ras mutation were sensitized by LY294002.$^{[33]}$ Our results given here seem to contradict with their data because we used normal human cells; however, we have to consider several factors for a fare comparison of these data. First of all, the cell lines are different; we used karyotypically normal human primary fibroblasts (Okayasu and J. Robinson, personal communication), and they used bladder carcinoma cells as well as rat cells. If one uses cancer cells, there is a possibility that various genes are affected without ras mutation, which could lead to a different phenotype. We have another normal human fibroblast cell line that was also radiosensitized, and the chromosome break repair was inhibited by LY294002 (Takakura and Okayasu, personal communication). The drug dose is another critical factor; with less than 10 $\mu$M of LY294002, the DNA-PK activity was not totally inhibited (K value for DNA-PK of LY294002 is 6 $\mu$M$^{[15]}$), but the PI3K activity is likely to be eliminated at 5–10 $\mu$M of LY294002. Thus, with low concentrations of LY294002, the PI3K pathway may play a role in radiosensitization by this drug. At LY294002 doses of 50 $\mu$M or higher, it is very likely that DNA-PK is affected, leading to the inhibition of radiation-induced DSB rejoining.

In summary, we have demonstrated the radiosensitizing effect of a PI3K inhibitor, LY294002, in irradiated normal human cells; at high LY294002 doses (50 $\mu$M or higher) this phenomenon is likely to be the result of DNA DSB repair inhibition by the drug. The DSB repair inhibition is clearly reflected in the excess number of interphase chromosome fragments in irradiated cells pretreated with LY294002. There is a good correlation between the level of radiation cell killing and the excess number of chromosome fragments of the G1 type. These findings along with the data by other groups could help develop a new class of radiosensitizer targeting the inhibition of NHEJ-type DSB repair.

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**REFERENCES**

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